PATENT

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Signature

Appl No.	: 10/479,080	Confirmation No. 1948
Applicant	: Michael Täger, et al.	
Filed	: November 26, 2003	
Title	: MEDICAMENT CONTAINING AN EFFECTOR OF THE GLUTATHIONE METABOLISM TOGETHER WITH ALPHA-LIPOIC ACID FOR USE IN KIDNEY REPLACEMENT THERAPY	
TC/A.U.	: 1626	
Examiner	: Taofiq A. Solola	
Docket No.	: 51494/M521	
Customer No.	: 23363	

DECLARATION OF MICHAEL TÄGER UNDER 37 CFR § 1.132

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November 14, 2006

Commissioner:

I, Michael Täger, hereby declare that:

1. I am a named inventor of the invention disclosed and claimed in the subject patent application, U.S. Patent Application Serial No. 10/479,080. I received a PhD degree in Medicine (german Dr. med.) from Otto-von-Guericke-University, Magdeburg, in German in 1994. I have not been employed by Serumwerk Bemberg AG. I hold a CEO position at IMTM GmbH, Germany. My responsibilities include scientific and financial management and I consider myself an expert in this area.

2. I have read the Final Rejection mailed July 14, 2006, and the prior art that has been cited by the patent examiner of the United States Patent and Trademark Office against the subject patent application.

3. The Examiner appears to have equated the treating of a disturbance of **thiol-disulfide status** with the correcting of glutathione (GSH) deficiency, yet the two medical conditions are not identical. Further, the prior art relied on by the Examiner is primarily directed to **free thiol content** instead of total thiol content. Additionally, it appears that the Examiner fails to recognize and understand the **synergistic effect** disclosed in the application when α-lipoic acid is used in combination with ambroxol, captopril, enalapril or ramipril. The remainder of this declaration will address:

- Differences between thiol-disulfide status and GSH deficiency;
- Failure of the cited prior art to disclose the problem of influencing the protein-bound thiol content of cells or tissues; and
- Unexpected, synergistic (super-additive) effects of the present invention.

To support the super-additive effect, additional experimental data showing the super-additive effect of the present invention will also be presented.

Thiol-Disulfide Status vs. GSH Deficiency

4. The present application is directed to a method of treating the thiol-disulfide status of a patient in kidney replacement therapy. The thiol status in cells and tissue is defined as the sum of protein-bound and non-protein-bound thiol groups. (See pages 1200-1202 in Schafer and Buettner, Free Radical Biol. Med. 2001, 30:1191-1212). Thus, the **total thiol content** of a cell is the sum of free thiols and protein-bound thiols. Protein-bound thiols are mainly present in the form of proteins containing sulfhydryl groups (-SH groups). The protein-bound thiols are detected as intracellular proteins as well as membrane-bound proteins.

5. A typical example of a non-protein-bound species is gluthathione, which counts for about 80% of the free or non-protein-bound thiol-groups detectable in a cell. The predominant fraction of free thiol-groups is represented by protein-bound thiol-groups, especially the amino acid cystein. These proteins are the main component in cellular development and differentiation processes, as well as in detoxification processes. Reduced gluthathione (GSH deficiency) occurs as the main intracellular free thiol compounds reaches an intracellular concentration between 2 - 10 mM, whereas the total concentration of protein-bound thiol groups is between 15 - 25 mM, as observed in erythrocytes (Rossi et al., Biochim. Biophys. Acta, 1995, 1243:230-238, page 230, column 1). Thus, the content of free thiol groups adds up to a total concentration of 12.5 mM, whereas protein-bound thiol groups are present in the cells up to a concentration of 25 mM. Thus, there is an excess of protein-bound thiol groups in a cell.

6. A thiol or a free thiol in a cell is typically discussed as an R-SH species, where R can be a protein, glutathione, cystein, or another group. The thiol is present in its reduced form. If the thiol group undergoes oxidation (for instance, in the presence of an oxidizing agent), a disulfide according to the formula R-S-S-R is formed. However, disulfides are not capable of reducing damaging compounds that may be present in a cell. It is therefore desirable to keep the concentration of disulfides in a cell 100 to 1000 times lower than the thiol-concentration. The negative influence of a low thiol level on several disease patterns is discussed at pages 1-5 of the application.

7. As previously mentioned, the physiological content of free thiols in cells and tissue is defined as the sum of protein-bound and non-protein-bound thiol groups (see pages 1200-1202 in Schafer and Buettner, Free Radical Biol.Med. 2001, 30: 1191-1212). On page 1200, column 2 the authors describe the concentration of protein-bound groups in cells and tissues as being much greater than that of reduced glutathione. They further state on page 1202, column 2, that the protein-bound SH groups can play a role in the antioxidant network of cells and thereby influence the redox-environment of the cell.

8. Further evidence of the reduced influence or contribution of free GSH on the total thiol status of a cell can be drawn from the fact that the thiol content of a cell is only reduced about 20 % after selective inhibition of the GSH synthesis (Täger et al., FRBM, 29, 1160-1165, 2000). Measurements of the total thiol content in cell samples were conducted before and after the addition of buthionin sulfoximin (BSO) – a known inhibitor of the glutathione synthesis. Table 2, column 2, of the Täger et al. reference shows the thiol expression indices as calculated by the ratio of thiol content of BSO-treated (GSH depleted) and untreated samples. Depending on the measuring method that is used, it can be clearly seen that the inhibition of glutathione synthesis only reduced the total thiol expression to a rather small extent.

9. Experimentally, it is possible to determine the total amount of free thiol-groups in a cell or even the amount of a specific thiol-group-containing compound, e.g. glutathione. In the present invention, however, the **total** amount **of free protein-bound and non-protein-bound thiol-groups** was determined.

10. One embodiment of the present invention treats the disturbed thiol status of a cell or tissue caused by a disturbance of the **total thiol content** (i.e., a disturbance of the thiol-disulfide status) by co-administration of α-lipoic acid (and/or a salt thereof) and a glutathione metabolism effector such as ambroxole (and/or salt) or an ACE inhibitor such as captopril, enalapril, ramipril, etc.. Tables 1 and 2 and Figures 1a,b and 2a,b of the pending application show specifically the influence of a combination of α-lipoic acid and ambroxole, captopril, or enalapril on the intracellular thiol status and the membrane-bound thiol status, respectively.

11. In summary, it is my opinion that a disturbance of thiol-disulfide status is not the same as a GSH deficiency and, as discussed in the next section, that one of skill in the art would not have been motivated to combine the cited references to arrive at the claimed invention

Prior Art Is Directed To Free Thiols and Does Not Suggest the Claimed Invention

12. Gillissen et al. relates to the anti-oxidative and anti-inflammatory properties of ambroxole *in vitro* and *in vivo* in animals and humans. **Gillissen et al. does not indicate an effect of ambroxole on the total amount of free protein-bound and non-protein-bound thiol groups.** It merely gives an indication of the specific influence of ambroxole on the GSH-metabolism. On page 612, column 1, last paragraph of Gillissen et al., only the general importance of the glutathione redox cycle is mentioned, without any link to a specific influence of ambroxole. On pages 616-618, the anti-oxidant activity of ambroxole as a radical scavenger and as a lipid antioxidant is described, but no direct effect of ambroxole on GSH is provided.

13. Derick et al. refers to the increase of intracellular, **unbound** thiols in a human T-cell line caused by administering α-lipoic acid. This increase is due to a rise in reduced glutathione (GSH) and reduced α-lipoic acid (DHLA). The amount of **protein-bound** thiol is not even considered in Derick et al. Further, Derick et al. does not discuss the influence of α-lipoic acid on the total thiol-disulfide status or the dependence of specific cellular functions on the thiol-disulfide status. Derick et al. also does not refer to a kidney replacement therapy characterized by a disturbed thiol-disulfide status.

14. Martin-Marteo et al. teaches the detection of oxidative stress in patients undergoing kidney replacement therapy by measuring the level of GSH and GSSG in the blood. It is stated that under these conditions, reduced GSH is not available to red-cell membranes in sufficient quantities to avoid free-radical damage. However, as stated above, an indirect correlation of oxidative stress and free thiol content is not generally applicable.

15. It would not be obvious to combine Gillissen et al., Martin-Marteo et al., and Derick et al. to achieve the present invention. Martin-Marteo et al. indicates that a reduced GSH level may lead to an increase of free-radical damage, but does not indicate how to treat the oxidative stress in a patient during kidney replacement therapy. Gillissen et al. only teaches a general function of

ambroxole as anti-oxidative agent, whereas Derick et al. describes the effect of α-lipoic acid on the GSH status of a specific cell line. None of the references mentions the importance of the complete thiol level in a cell characterized by the sum of all protein-bound and non-protein-bound thiol groups for a dysfunction of a cell or a tissue. Furthermore, none of the references provides information about **how** to increase the total amount of free thiol groups in a cell or tissue. Therefore, a person skilled in the art would have had no motivation to combine the references in order to come to the beneficial solution of the claimed invention. Furthermore, no reference suggests that a synergistic effect can be had by combining, e.g., α-lipoic acid and ambroxole in a medicament.

16. Moreover, even if Gillissen et al. and Derick et al. were combined, a person skilled in the art would not come to the beneficial solution of a medicament according to pending Claim 1. According to Gillissen et al., ambroxole is applied as a radical scavenger *in vitro* and *in vivo*, and α-lipoic acid is used in Derick et al. for limited increasing the non-protein-bound thiol level in the form of GSH in a T-cell line. Neither reference teaches or suggests a medicament or method for the treatment of a disturbed cellular thiol-disulfide status in a kidney replacement therapy by increasing the total **protein-bound and non-protein-bound thiol** amount in a cell according to the pending claims.

17. Elena et al. relates to the anti-oxidative properties of the ACE-inhibitors enalapril and captopril due to their influence on the metabolism of compounds with non-protein-bound thiol groups, specifically GSH. The effect was determined by measuring the total amount of GSH and its disulfide GSSG in mouse tissue. Since separate determination of the glutathione amount was carried out, it is not possible to anticipate the ratio of the oxidized and reduced glutathione form. Therefore, it is inappropriate to conclude that the amount of free GSH increased due to the action of ACE inhibitors. It is even possible that the increase of GSH / GSSG is only caused by an increase of GSSG levels. Elena et al. does not provide any evidence for an increase of GSH. Elena et al. also does not refer to the importance of all free protein-bound and non-protein-bound thiol groups in a cell or tissue.

18. A combination of Derick et al., Elena et al. and Martin-Marteo et al. would not lead a skilled person to the beneficial solution of using a combination of an ACE-inhibitor and α-lipoic acid for treatment of the thiol status of a cell. None of the documents refers to the impact of the total amount of free thiol groups in kidney replacement therapy, let alone provides a solution of how to solve the problem.

19. In summary, it is my opinion that:

- No document teaches the influence of any of the claimed compounds on the protein-bound thiol groups;
- No document teaches the application of the combined drug preparations of the present invention;
- No document teaches the synergistic, super-additive effect of the combined drug preparations of the present invention; and
- The skilled person would not have been motivated to combine the references as suggested by the U.S. patent examiner, and even if so combined, the references would not suggest the present invention.

Super-Additive Effect

20. The Examiner states on page 7 of the Final Rejection that "[t]here is no clear demonstration of synergistic effect from these results." In my opinion, the Examiner is in error because he did not take into account the effect shown for the control group when calculating the effectiveness of combining drugs.

21. As one example, the super-additive effect is demonstrated in the case of the membrane-bound thiol expression. Table 2 on page 18 of the specification shows the influence of α-lipoic acid, capropril, and a combination of the two drugs on the total expression of membrane-bound thiol. After 14 days of cultivation, the singular administration of α-lipoic acid caused a 27%

increase of the thiol expression: ((1.34-0.98)/1.34x100) = 27%, whereas the singular administration of captopril caused a 1% increase: ((0.99-0.98)/0.99x100) = 1%. Note that 0.98 is the effect shown by the control. When the two drugs were administered in combination, the effect (increase of membrane-bound thiol expression) was **NOT** (27+1) = 28%. Rather, the effect of the combination was 58%: ((2.36-0.98)/2.36x100) = 58%. This difference -- 58% vs. 28% -- is a remarkable, super-additive effect. This super-additive effect on the membrane-bound thiol expression and, hence, the total thiol expression of a cell, is striking, unexpected, and cannot be predicted from the prior art.

Additional Data Demonstrating the Super-Additive Effect

Influence on the Cellular Thiol Status of Monocytes in Mice

22. The influence of the combination of both drugs, α -lipoic acid and ambroxole, was investigated *in vivo*. Balb-c mice were put into a state of thiol deficiency by keeping them on a strict, thiol-free diet. This model shows typical conditions of renal replacement therapy. The induction of thiol deficiency was controlled by the determination of the intracellular thiol content at the individual cell level. This was carried out using 5-chloromethylfluorescein diacetate in flow cytofluorimetry. Thiol deficiency was demonstrated after 10 days on the specific diet. The artificially thiol-depleted mice were treated with α -lipoic acid, ambroxole, and the combination of both drugs. Untreated thiol-deficient and healthy animals were used as controls. The drugs were administered orally in the drinking water over a length of time of 52 days. For each study group the investigations were repeated 5 times with 5 animals (n=25). In the appended Figure 8, the results of the estimated intracellular thiol content of monocytes from peripheral blood are shown. The data are shown as the ratio of the mean cellular fluorescence intensity for treated or untreated thiol deficient animals to the parallel estimated healthy animals in accordance with following equation:

TEI (thiol expression index)= mfi_{test} / $mfi_{healthy}$ x 100. (mfi: mean fluorescence intensity)

23. The treatment of thiol-deficient mice with α-lipoic acid or ambroxole alone had no significant effect on the intracellular thiol content of monocytes (Fig. 8A,B). Under the treatment of the combination of α -lipoic acid and ambroxole, beginning after 14 days, a significant improvement of intracellular thiol content occurred (Fig. 8C). This effect was significant and super-additive. A maximum in the super-additive action was achieved after treatment period of 28 days, which was constant during the remaining course of the experiment. It was particularly obvious that the administration of the individual substances did not show a significant influence at any point during treatment period.

24. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed this 28 day of NOVEMBER 2006

By: _____

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